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## ARTICLE

# The altered expression of $\alpha 1$ and $\beta 3$ subunits of the gamma-aminobutyric acid A receptor is related to the hepatitis C virus infection

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**Abstract** The modulation of the gamma-aminobutyric acid type A (GABA A) receptors activity was observed in several chronic hepatitis failures, including hepatitis C. The expression of GABA A receptor subunits  $\alpha 1$  and  $\beta 3$  was detected in peripheral blood mononuclear cells (PBMCs) originated from healthy donors. The aim of the study was to evaluate if GABA A  $\alpha 1$  and  $\beta 3$  expression can also be observed in PBMCs from chronic hepatitis C (CHC) patients and to evaluate a possible association between their expression and the course of hepatitis C virus (HCV) infection. GABA A  $\alpha 1$ - and  $\beta 3$ -specific mRNAs presence and a protein expression in PBMCs from healthy donors and CHC patients were screened by reverse transcription polymerase chain reaction (RT-PCR) and Western blot, respectively. In patients, HCV RNA was determined in sera and PBMCs. It was shown that GABA A  $\alpha 1$  and  $\beta 3$  expression was significantly different in PBMCs from CHC patients and healthy donors. In comparison to healthy donors, CHC patients were found to present an increase in the expression of GABA A  $\alpha 1$  subunit and a decrease in the expression of  $\beta 3$  subunit in their PBMCs. The modulation of  $\alpha 1$  and  $\beta 3$  GABA A receptors subunits expression in PBMCs may be associated with ongoing or past HCV infection.

## Introduction

The gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the mammalian central nervous system, causes changes in a polarization of cell membrane acting through the activation of GABA receptors. The most prevalent, GABA type A (GABA A), receptors tend to exist as pentameric structures consisting of various combinations of six major subunits:  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\pi$  [1]. Recent data indicate that GABAergic activity is not restricted to the central nervous system, but also involves cells of different origin that, like hepatocytes, possess the peripheral type of GABA A receptors [2]. As was shown in several studies, an activation of GABA A receptors (especially the  $\beta 3$  subunit) leads to the hyperpolarization of cell membrane, which, in turn, causes a rapid decrease in cell proliferation [3, 4]. This feature seems to be interesting with regard to the study that demonstrated the impact of cell polarization on the efficiency of hepatitis C virus (HCV) entry [5]. An elevated GABAergic activity was found to be responsible for the impaired hepatocyte proliferation in regenerating livers after partial hepatectomy [6, 7]. On the other hand, it has been known since the early 1980s that the serum level of GABA may be elevated in case of acute or chronic hepatocellular failure [8], and the GABA neurotransmitter system is involved in the pathogenesis of hepatic encephalopathy (HE) in humans [9]. Recently, it has been suggested that HE-dependent ammonia may be developed due to the modification of the GABA A receptor affinity [10]. Other findings suggest that increased inhibition through GABA A receptors may represent an important pathophysiological mechanism of fatigue in chronic HCV infection [11].

This multifunctionality of GABAergic action in numerous liver failures has drawn our attention to the possible role of the modulation of GABA A receptors expression in the course of

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HCV infection and in the response to the antiviral treatment. Although the liver is the main place of HCV replication, gathered data, including our own [12, 13], indicate that HCV can persist and replicate efficiently in extrahepatic tissue, including peripheral blood mononuclear cells (PBMCs). HCV RNA can persist in PBMCs long after spontaneous or treatment-induced viral elimination from sera [14], but the relevance of this phenomenon is still unknown. It has been documented recently that PBMCs originated from the healthy human population express functional  $\alpha 1$  and  $\beta 3$  subunits of the GABA A receptor [15]. The aim of the current study was to investigate whether the comparable expression of GABA A subunits can be observed in PBMCs from chronic hepatitis C (CHC) patients that have undergone anti-HCV treatment. Consequently, not only did we succeed to show  $\alpha 1$  and  $\beta 3$  expression in PBMCs from HCV-infected patients, but our results also demonstrated the substantial differences in  $\beta 3$  and, less manifested, in  $\alpha 1$  subunits expression in PBMCs between healthy donors and post-treatment HCV patients. We then speculate on how the alterations in the expression of GABA A subunits may be of special importance for HCV RNA persistence.

## Materials and methods

Blood samples were collected, after the informed consent had been obtained, from ten healthy donors (6 males, 4 females: age 18–26 years) and from 22 chronically infected with HCV patients (12 males, 10 females: age 16–22 years) within 2 weeks after the cessation of antiviral treatment (IFN alfa2b+ribavirin). Neither patients nor healthy controls were taking medication known to alter GABA A receptor expression or activity. PBMCs and sera were isolated by blood centrifugation in density gradient (Histopaque 1077, Sigma). Total RNA

was extracted from PBMCs by a modified guanidinium thiocyanate/phenol/chloroform technique.

HCV RNA presence in sera was determined by reverse transcription polymerase chain reaction (RT-PCR) (Cobas, Amplicor HCV 2.0 Monitor, Roche). HCV RNA in PBMCs was detected by RT-PCR as described previously [13]. Briefly, 6  $\mu$ g of total RNA were reverse-transcribed and amplified by MasterAmp™ Tth DNA Polymerases (Epicentre® Biotechnologies) with external HCV-specific primers (Table 1) in a reaction as follows: 20 min of RT at 70°C and 3 min at 94°C, followed by 35 cycles of 94°C for 20s, 50°C for 20s, 72°C for 20s, and 72°C for 7 min. The resultant amplicon was used in the second-round PCR (2 min at 94°C, 30 cycles of 94°C for 40s, 55°C for 40s, 72°C for 40s, and 72°C for 10 min). After PA gel electrophoresis, an HCV-specific product of 278 bp was visualized with ethidium bromide staining.

For the detection of mRNAs specific for GABA A receptor subunits  $\alpha 1$  and  $\beta 3$ , random cDNA was synthesized according to the manufacturer's instructions by using the Transcription High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) and then amplified in "nested" PCR [15] with gene-specific primers (Table 1). The expression analysis of actin was used as an internal control for each sample. PCR assays (each of 35 cycles) were performed as follows: 5 min of preliminary heating at 94°C, 94°C for 40s, 50°C (for GABA A  $\alpha 1/\beta 3$ )/55°C (for actin) for 40s, 72°C for 40s, and 72°C for 10 min. PCR  $\alpha 1$ - and  $\beta 3$ -specific products of 95 bp and 110 bp, respectively, were analyzed as described above.

GABA A  $\alpha 1$  and  $\beta 3$  subunit expression was determined by Western blotting. Protein lysates (20  $\mu$ g/lane) were separated on 10% SDS-PA gel. Resolved proteins were electroblotted into nitrocellulose and incubated with GABA A  $\alpha 1$ , 52 kDa (GTX 30204, dilution 1:12,000) and  $\beta 3$ ,

**Table 1** List and sequences of primers used in the reverse transcription polymerase chain reaction (RT-PCR) analysis and size of PCR products

Specificity of primers	PCR product (bp)	Sequence of forward and reverse primers
HCV RNA (external)	321	F: CCACCATGAATCACTCCCCTGT R: GCTCATGGTGCACGGTCTACGAGACCT
HCV RNA (internal)	278	F: GTCTTCACGCAGAAAGCGTCTAGCC R: CACTCGCAAGCACCTATCAGGCAG
GABA A $\alpha 1$ (external)	241	F: CGGTCAATTTTGCTGACACT R: GGTATGCATGGGATGGC
GABA A $\alpha 1$ (internal)	95	F: GACCTCTTTAAGGTTCTATGG R: GCTCCAACAGCAACCAGC
GABA A $\beta 3$ (external)	319	F: CACATCGGTTAGATCAGG R: CAAGGCAAAGAATGACCG
GABA A $\beta 3$ (internal)	110	F: CGCTGGAAGTTCACAATG R: CGAGGCATGCTCTGTTTC
$\beta$ -Actin	434	F: CAAAGACCTGTACGCCAACACA R: AACCGACTGCTGTACCTTCAC

55 kDa (GTX 261302, dilution 1:2,000) specific antibodies purchased from Gene Tex, Inc. The reactions with the goat antibodies against  $\beta$ -actin, 43 kDa (SC-1615, Santa Cruz Biotechnology) were carried out at the dilution of 1 to 300. The bound antibodies were visualized using the enhanced chemiluminescence (ECL) Western blotting reagent (SC-2048, Santa Cruz Biotechnology) with signals captured on film. In order to quantify the density of signals, the Bio-Rad Quantity One system was used. To estimate the comparative levels of GABA A  $\alpha$ 1 and  $\beta$ 3 subunits expression, all immunoreactivities were normalized to  $\beta$ -actin expression before statistical analysis.

The statistical analysis was conducted using Statistica 8.0 PL Software (StatSoft). The results for groups were compared using the Kruskal–Wallis test and the Mann–Whitney test.  $p$ -values < 0.05 were considered to be statistically significant.

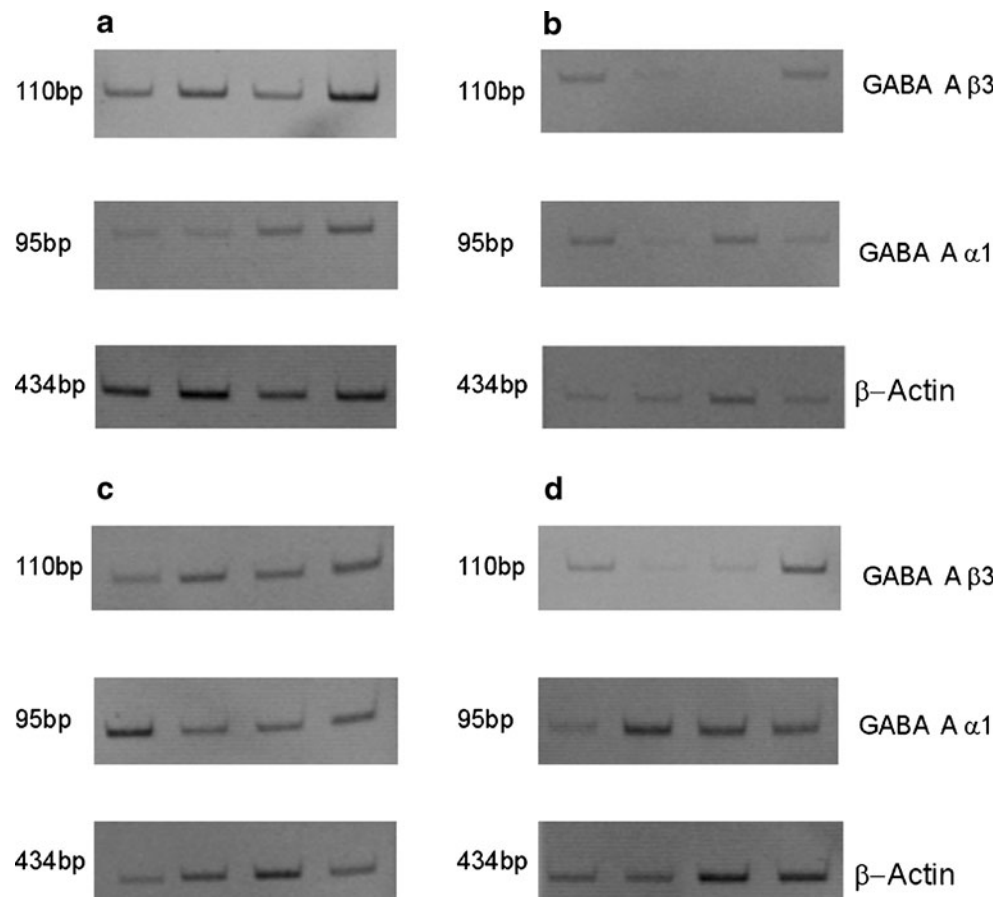
## Results

mRNAs of both  $\alpha$ 1 and  $\beta$ 3 subunits of GABA A receptor are expressed in human PBMCs

The presence of mRNAs of  $\alpha$ 1 and  $\beta$ 3 was evaluated in PBMCs. Cells were collected from healthy donors and

CHC patients who had undergone antiviral treatment. According to the HCV RNA status in sera and in PBMCs, three subgroups of patients were distinguished: 0/0 with sera and PBMCs free from HCV RNA ( $n=10$ ); 0/1 with sera negative for HCV RNA, but with HCV RNA-positive PBMCs ( $n=6$ ); and the 1/1 group, where sera and PBMC samples contained HCV RNA ( $n=6$ ). To analyze the gene expression of GABA A receptor subunits  $\alpha$ 1 and  $\beta$ 3, reverse transcription followed by nested PCR was used. PCR amplification produced single  $\alpha$ 1- and  $\beta$ 3-specific PCR products, of 95 and 110 bp, respectively. Our results showed that all PBMC samples contained mRNA specific for the  $\alpha$ 1 and  $\beta$ 3 subunits of GABA A receptor. The expression of  $\beta$ -actin was used as the endogenous control of gene expression. As shown in Fig. 1, the gene expression of GABA A receptor subunits differs among PBMC samples, even among the same group. No significant differences were found in gene expression level for either  $\alpha$ 1 or  $\beta$ 3 between groups. We could only observe that  $\beta$ 3 gene expression showed a margin tendency to be elevated in PBMCs from healthy donors in comparison to the HCV-infected patients. In contrast, the gene expression of  $\alpha$ 1 subunit in PBMCs tends to be slightly higher in the group of CHC patients compared to the healthy controls.

**Fig. 1** Gamma-aminobutyric acid type A (GABA A) receptor,  $\alpha$ 1 and  $\beta$ 3 genes expression in peripheral blood mononuclear cells (PBMCs). The figure shows several examples of the results obtained by reverse transcription polymerase chain reaction (RT-PCR) analysis performed in the groups as follows: **a** healthy donors (HD) and **(b–d)** anti-hepatitis C virus (HCV)-treated chronic hepatitis C (CHC) patients, who: **b** eliminated HCV RNA from both sera and PBMCs (0/0), **c** only from sera (0/1), or **d** neither from sera nor from PBMCs (1/1). As a control of gene expression, the analysis of  $\beta$ -actin was performed



## Expression analysis of $\alpha 1$ and $\beta 3$ subunits of GABA A receptor in PBMCs at the protein level

The presence of the protein expression of GABA A  $\alpha 1$  and  $\beta 3$  subunits was confirmed using Western blot in all of the tested PBMC samples. Figure 2 shows several examples of Western blot analysis performed on lysates obtained from the PBMCs of healthy donors and CHC patients. The quantitative immunoblotting based on a density analysis, described in the **Materials and methods** section, was utilized in order to determine the possible differences in the GABA A  $\alpha 1$  and  $\beta 3$  subunits expression between PBMC samples. As indicated in Fig. 3a, the GABA A  $\alpha 1$  protein expression was somewhat higher ( $p=0.047$ ) in the group of anti-HCV-treated CHC patients than in the group of healthy controls. However, no statistical differences were found between individual subgroups of patients.

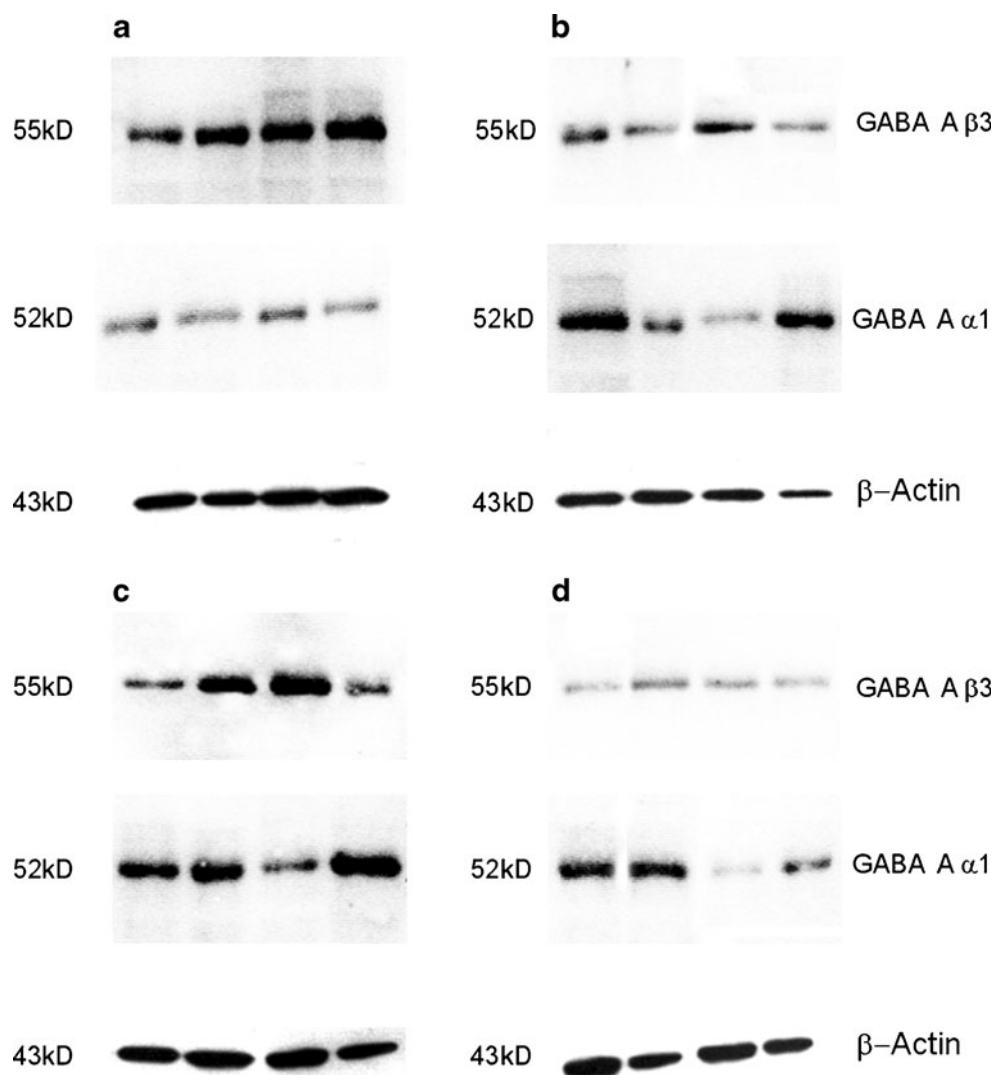
An opposite relation was found for the expression of  $\beta 3$  subunit. In PBMCs from anti-HCV-treated CHC patients, the

$\beta 3$  protein showed decreased levels in comparison to the group of healthy donors. As presented in Fig. 3b, significant differences ( $p=0.0038$ ) in the  $\beta 3$  expression among healthy donors and the three subgroups of CHC patients were observed as a result of the  $\beta 3$  expression analysis. The lowest expression level of  $\beta 3$  protein, compared to the healthy donors, was observed in PBMCs from patients, who, despite the antiviral treatment, have retained HCV RNA in both sera and PBMCs ( $p=0.00047$ ).

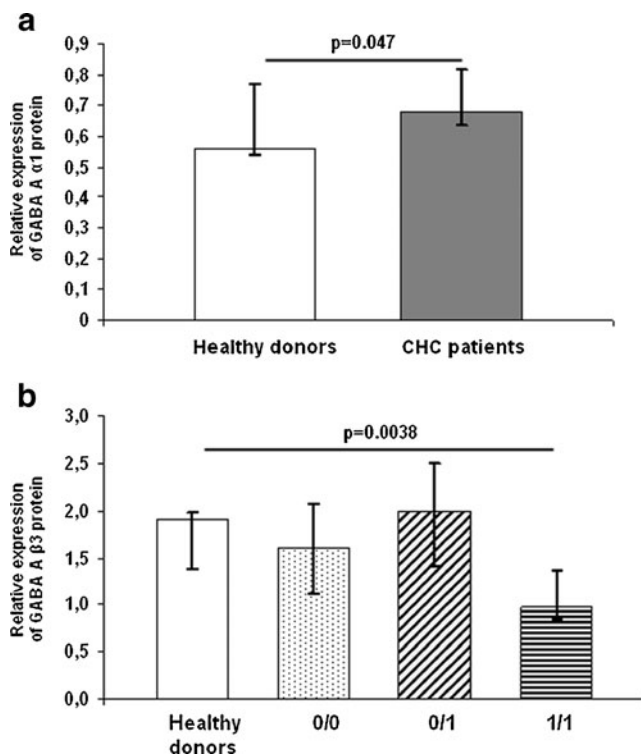
## Discussion

Several studies have revealed the contribution of a variety of host factors to the development of HCV RNA persistence in chronically infected patients, despite having used antiviral treatment [13, 16, 17]. Our study was designed to evaluate whether the expression of chosen subunits of GABA A receptors in PBMCs bears any relation to HCV infection and/

**Fig. 2** Expression of  $\alpha 1$  and  $\beta 3$  subunits of the GABA A receptor in PBMCs as revealed by Western blot analysis. Representative images of Western blot analysis come from groups as follows: **a** healthy donors (HD) and **(b–d)** anti-HCV-treated CHC patients, who: **b** eliminated HCV RNA from both sera and PBMCs (0/0), **c** only from sera (0/1), or **d** neither from sera nor from PBMCs (1/1). The analysis of  $\beta$ -actin was performed as a control of gene expression







**Fig. 3** Differences in GABA A  $\alpha 1$  and  $\beta 3$  subunits expression in PBMCs evaluated by Western blot analysis. The results are shown after normalization to the  $\beta$ -actin expression. **a** Relative expression level of GABA A  $\alpha 1$  protein in PBMCs from healthy donors and from post-treatment CHC patients. **b** Relative expression level of GABA A  $\beta 3$  protein expression in PBMCs from healthy donors and three groups of anti-HCV-treated CHC patients (0/0, 0/1, 1/1)

or the path of HCV RNA elimination after anti-viral treatment. Studies that were carried out on other viruses like herpes simplex virus (HSV) and human immunodeficiency virus (HIV) indicated that the expression of GABA A receptor on the target cells could be modulated upon the viral infection [18, 19]. Although GABA A receptors tend to exist as a pentameric structure consisting of six major GABA A receptor subunits [1], we decided to screen the expression of two of them:  $\alpha 1$  and  $\beta 3$ . This choice was grounded on Alam et al.'s study [15] that confirmed the significant content of the  $\alpha 1$  subunit in PBMCs and fractionated T-cell populations. The evaluation of  $\beta 3$  expression, despite the lower abundance of this subunit in PBMCs, seemed interesting due to the confirmed impact on a proliferative activity of other cells [20, 21].

If governing the HCV infection indeed plays any role in the GABA A receptor activity, the modulation of their expression would be an expected phenomenon. To address this issue, we analyzed the  $\alpha 1$  and  $\beta 3$  GABA A expression in PBMCs using nested PCR and quantitative immunoblotting. Our results showed, for the first time, that the expression of the  $\alpha 1$  and  $\beta 3$  subunits of the GABA A receptor is common not only for healthy donors (15), but also for anti-HCV-treated

patients. Moreover, the expression of these subunits at the protein level displayed differences between healthy donors and CHC patients. A marginally significant elevation of  $\alpha 1$  GABA A in PBMCs was demonstrated for anti-HCV-treated patients when compared with healthy donors. It seems interesting that the expression of  $\alpha 1$  GABA A receptor subunit is detectable in the majority of PBMCs subtypes, such as: T-cells, B-cells, and monocytes, in other words, in cells that are also able to maintain HCV during chronic infection [22]. Taking into account that the increased expression of these receptors in T-cells downregulates the effector T-cell response [23, 24] and that the defective function of HCV-specific T-cells contribute to the chronicity of infection [25–27], we can hypothesize that GABA A activation may contribute to the impaired response to the HCV infection.

In contrast, the majority of PBMCs from the anti-HCV-treated patients represented significantly lower expression of the  $\beta 3$  subunit of the GABA A receptor than the healthy donors' PBMCs. Thus, also in case of the  $\beta 3$  subunit, expression measured at the protein level does not reflect precisely the gene expression level. This type of discrepancy, probably connected with the post-transcriptional regulation, was observed for GABA A receptor expression previously [28]. The lowest level of GABA A  $\beta 3$  receptor expression was observed in these PBMCs, where HCV RNA presence in cells was accompanied by the HCV RNA positivity of sera. As was demonstrated, the transfection of hepatoma cells with  $\beta 3$ -specific cDNA resulted in a significant decrease of cell proliferation [29]. Similarly, an increased GABA A  $\beta 3$  receptor expression was found to act as an inhibitory signal for hepatic cell proliferation, whereas the downregulation of the GABA A  $\beta 3$  receptor expression was observed in malignant hepatocyte cell lines [4]. Although less is known about the role of  $\beta 3$  subunit expression of the GABA A receptor in PBMCs, we can hypothesize that, like in the case of hepatic cells, the decreased expression of GABA A  $\beta 3$  receptor in PBMCs may alter the proliferative activity of these cells. Since HCV-infected cells present enhanced proliferation in comparison to non-infected cells [30, 31], this process appears to play an important role in HCV RNA replication. On the other hand, it was previously demonstrated that the drug-dependent inhibition of GABA A receptors expression does not alter HCV load [32], which may suggest that, rather, HCV infection is responsible for inducing such changes in GABA A expression that favor HCV propagation in target cells.

In conclusion, the current study provides evidence for the  $\alpha 1$  and  $\beta 3$  expression in PBMCs from HCV-infected patients. Decreased GABA A  $\beta 3$  expression, which is observed in HCV RNA-positive PBMCs, may create a favorable environment for HCV RNA persistence. Future studies should elucidate whether the alteration of GABA A expression, which is observed during CHC infection,

has an impact on the development of hepatocellular carcinoma.

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